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Pentacyclic Triterpenoids from Olive Fruit and Leaf

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This work establishes a new procedure for the extraction and analysis of pentacyclic triterpenes, with which fruits and leaves from three Spanish olive cultivars ("Picual", "Hojiblanca", and "Arbequina") has been studied. The leaf contains important amounts of oleanolic acid (3.0−3.5% DW), followed by significant concentrations of maslinic acid and minor levels of ursolic acid, erythrodiol, and uvaol. The abundance and profile of triterpenoids change during the leaf ontogeny. In the fruit, triterpenes are exclusively located in the epicarp at concentrations 30-fold lower than that in the leaf. Maslinic acid is the main triterpenoid, only accompanied of oleanolic acid. Along the ripening the levels of these triterpenes decreased. All the analyzed leaves and fruits come from the same agricultural estate, with identical climate and culturing conditions. For this reason, the found differences could majorly be attributable to the genetic factors of the olive cultivars.

KEYWORDS: Olive (*Olea europaea* L.) leaf and fruit; pentacyclic triterpenoids; oleanolic acid; maslinic acid; erythrodiol; uvaol; ursolic acid

INTRODUCTION

The search for a healthier way of life has generated in the last two decades a rising attention for the obtaining of pentacyclic triterpenoids, plant bioactive compounds that are increasingly demanded by the markets because they could be introduced in new functional foods, drugs, or cosmetics.

Pentacyclic triterpenes are plant secondary metabolites biosynthesized by the acetate/mevalonate cytosolic pathway which yields (3*S*)-2,3-oxidosqualene (OS). When OS adopts a "chair−chair−chair−boat" conformation, the OS cyclases catalyze the synthesis of the different pentacyclic triterpene structures via a mechanism involving the generation of carbocationic intermediates (*1*).

Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid) and its isomeric, ursolic acid (3β-hydroxy-ursan-12-en-28-oic acid) (**Figure 1**), are two triterpenic compounds widely distributed in the plant kingdom as free acids or forming part of triterpenoid saponins. Besides their supposed antioxidant activity, these pentacyclic triterpenes are strongly considered for other pharmacological properties with beneficial effects for the health. There is evidence of the antiviral (including anti HIV), antibacterial, antifungal, anticariogenic, antiallergic, anti-inflammatory, hepatoprotector, gastroprotector, hypolipidemic, antiatherosclerotic, and antidiabetic effects (*2*−*4*). In addition, they interfere in several phases of the development of different types of cancer, disabling the genesis and evolution of tumors and inducing the apoptosis of the tumor cells (*5*−*7*). Recently, it has been reported that oleanolic acid also has beneficial effects on multiple sclerosis (*8*). Other pentacyclic triterpenes seem to share similar pharmacological activities.

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Olive (*Olea europaea* L.) fruit and leaf have been reported to contain a wide variety of sterols and triterpenoids in their epidermis (*9*). In both organs, triterpenic acids are found as free acids, whereas the pentacyclic triterpenols can be free or sterified with fatty acids (*10*). The analysis of the olive fruit cuticle waxes has demonstrated that the oleanane-type triterpenoids are predominant, with maslinic and oleanilic acid as the major triterpenes (*11*). The profile and concentration of the different triterpenoids seem to be significantly influenced by the fruit developmental stage (*12*).

The triterpenoid content in the olive leaf is higher than that in the fruit, and the oleanane-type derivatives are those that equally appear in major concentrations (*13*). Our research group has studied the composition of leaves from different olive cultivars and has observed that the triterpene contents are dependent on the variety (*14*, *15*). In any case, oleanolic acid is the major triterpenic component and appears in very important quantities as free acid (ca. 3% of the leaf dry weight) (*14*).

At the view of these precedents, the olive tree constitutes a suitable raw material for the recovery of oleanolic acid and other pentacyclic triterpenes. The aim of this work is to study the presence and relative concentrations of pentacyclic triterpenoids in fruits and leaves from the "Picual", "Hojiblanca", and "Arbequina", the three main Spanish olive cultivars. Two different developmental stages in fruits and five in leaves have been considered, and the distribution of triterpenoids among the different fruit tissues was investigated in the "Arbequina" variety.

MATERIALS AND METHODS

Plant Material, Reagents and Standards. Olive leaves and fruits of the "Picual", "Hojiblanca", and "Arbequina" cultivars were hand-picked from adult trees (> 10 years old) of the "Hacienda Guzmán" olive grove in La Rinconada (Seville, Spain).

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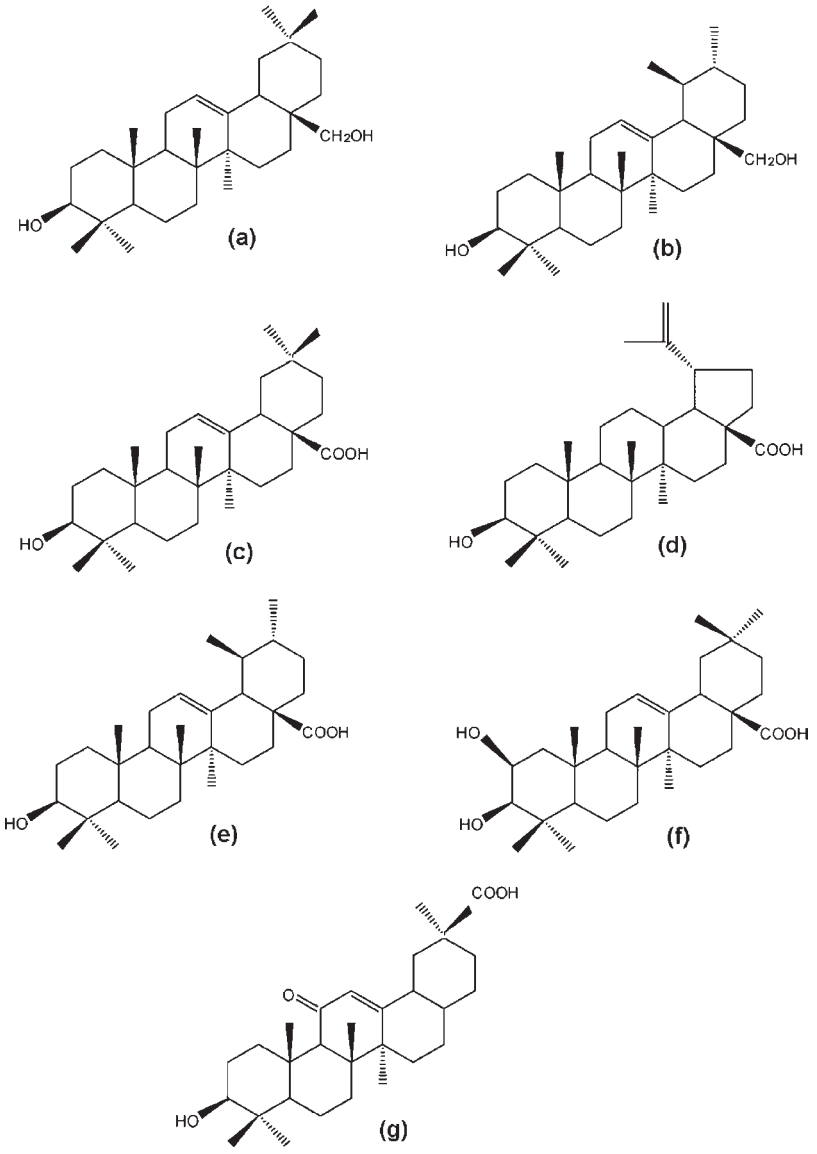

Figure 1. Chemical structures of the main triterpenic compounds found in olive fruits and leaves: (**a**) erythrodiol, (**b**) uvaol, (**c**) oleanolic acid, (**d**) betulinic acid, (**e**) ursolic acid, (**f**) maslinic acid, and (**g**) 18β-glycyrrhetinic acid used as internal standard.

Fresh leaves at five different stages of the ontogeny, according to their position in the olive twigs, were collected around the trees: stage 1 comprises the light-green leaves surrounding the apical bud, stage 2 includes olive leaves set in the distal area of the branch next to the apical bud, with a semimaximal size and bright light-green color, stage 3 congregates leaves set in the distal third of the branch, with dark-green color and semimaximal size, stage 4 group leaves with definitive color and size established in the second third of the olive twigs, and stage 5 includes adult presenescent leaves set in the proximal extreme of the branch. Two hundred and fifty leaves at each developmental stage (50 leaves from five different trees) were picked for each olive cultivar.

On the other hand, a representative sample of 250 healthy fruits of each olive cultivar (50 drupes from five different trees) were harvested around the trees at two different ripening times, corresponding to the "green mature" and "black ripe" stages. "Green mature" drupes correspond to fruits that achieved their definitive size and were physiologically mature to introduce into the ripening although their epidermis remained with an intense green color. "Black ripe" olives means fruits whose epidermis was fully purple–black colored. In addition to the visual inspection, the ripening stage was also objectively assessed by the skin color measurement. This color was individually determined in 100 fruits using a Minolta CR-200 colorimeter (Minolta, Japan), measuring the parameters L^*, a^*, and b^* of the Hunter's space of color. The results were expressed by the colorimetric index CI = $[L^*(b^* - a^*)]/100$, established by us in ref *16*, which perfectly monitors the skin color evolution from the intense green to the fully black stage following a descending sygmoidal Boltzman-type curve.

The distribution of pentacyclic triterpenes among the tissues that constitute the "Arbequina" fruit was also studied. Olive is a stone fruit that can be structurally separated in four different tissues: epicarp (skin), mesocarp (flesh), and woody endocarp (stone) containing the seed. Using a scalpel, we have proceeded to the careful isolation of the epicarp, mesocarp, and stone. Subsequently, the stone was cut throughout its minor diameter using a device designed and constructed in our institute, and the seed could be recovered whole and separated from the woody tissue.

All solvents used for triterpene extraction and analysis were of analytical grade. 18β-Glycyrrhetinic acid, hexamethyldisilazane, and trimethylchlorosilane were purchased from Sigma-Aldrich (Sigma-Aldrich Quimica, Madrid, Spain).

Extraction of Triterpenic Compounds. Fruits and leaves were washed with distilled water and dried in a convection heating furnace at 100 °C for 24–48 h until moisture loss (ca. 45% of the initial fresh weight). Then, 50 whole olive organs were twice extracted by maceration with 20 mL/g of absolute ethanol at 25 °C for 1 h with occasional shaking. The ethanolic extracts were obtained by filtration, collected, and stored at 5 °C until analysis. In the case of the whole olive fruits and prior to the ethanol extraction, a flash-washing with hexane (1 mL/g) was carried out.

When triterpene composition of the "Arbequina" fruit tissues was studied, 10.0 ± 0.1 g of epidermis or mesocarp were washed with hexane

and processed by maceration as mentioned above, whereas the endocarp and the seed were only extracted with absolute ethanol by crushing and homogenization in Sorvall Omni-mixer.

Derivatization of Triterpenoids. Because of the low volatility and high molecular weight of pentacyclic triterpenoids, derivatization prior to gas chromatography analysis is required. The silylating reagent was prepared by adding 3 mL of hexamethyldisilazane and 1 mL of trimethylchlorosilane to 9 mL of anhydrous pyridine. An aliquot of the ethanolic extracts (100 μL) and 100 μL of a 0.5 mg/mL solution of 18β-glycyrrhetinic acid as internal standard were placed in 1 mL gastight vials and evaporated to dryness under a N_2 stream. The residue was dissolved in 200 μL of the silylating reagent and heated at 80 °C for 2 h. After derivatization, an aliquot of the silylated solution was submitted to GC-FID analysis.

GC-FID Analysis. Identification and quantification of triterpenes were carried out by a novel modification of the method previously established in our lab (*17*). One μL of the silylated sample was injected in an Agilent 6890N GC (Agilent Technologies, CA), equipped with a Rtx-65TG Crossbond capillary column (30 m × 0.25 mm I.D.; 0.1 mm film thickness) coated with 35% dimethyl−65% diphenyl polysiloxane as stationary phase (Restek, Co., Bellefonte, PA) and a FID detector. The injection was realized in split mode, and hydrogen was used as carrier gas (pressure at column head 140 kPa). The oven temperature was initially established at 260 °C for 9 min and programmed to increase up to 280 °C at a rate of 10 °C/min and maintained at this value for 8 min. The injector and detector temperatures were isothermically established at 300 °C. All samples were analyzed in triplicate.

Statistical Analysis of Data. All data are presented as the means ± standard deviation from three independent experiments carried out in triplicates. The data were evaluated by one-way ANOVA with the SigmaPlot 11.0 software (SPSS Inc., Chicago, IL), and the differences between means were assessed using the Duncan's multiple-range test. Statistical significance was considered at $p > 0.05$.

RESULTS

Optimizing Triterpene Extraction and Analysis. A new procedure for extracting triterpenoids from olive fruits and leaves has been established as a evolution of those described by our research group (*17*, *18*). Previously to the ethanolic extraction, both olive organs and tissues were dried in a convection-heating furnace at 100 °C. This process, which did not lose analytes but removed the moisture, has been demonstrated to be useful to avoid the interference of pigments and other compounds that may be extracted together with pentacyclic triterpenes. Likewise, washing the olive fruits with hexane before the alcoholic extraction has been effective for eliminating acylglycerols and other lipids that interfere with the performance of the GC-FID analysis. All the hexanic wastes were collected, concentrated, and analyzed to confirm the absence of triterpenic compounds. On the other hand, we have observed that two consecutive extractions with absolute ethanol are effective for achieving the total recovery of triterpenes. When a third extraction was carried out to proof this item, none of the target triterpenoids was detected.

In this work, triterpenes have been analyzed by GC-FID using 18β-glycyrrhetinic acid as internal standard. Acute and symmetrical peaks for all the detected pentacyclic triterpenoids were obtained at the aforementioned chromatographic parameters. 18β-Glycyrrhetinic acid is an oleanane type pentacyclic triterpene which is present in liquorice roots, forming part of the triterpenoid saponin glycyrrhizic acid. Despite the structural analogy with the olive triterpenes (**Figure 1**), 18β-glycyrrhetinic acid exhibited, in the conditions that the analysis was run, a well distinguished retention time (RT≈13.8 min) that does not interfere the RTs of the olive triterpenoids (**Figure 2**). Calibration curves for this standard in the concentration range 0.2−2.0 mg/mL were determined. The amount/area response was linear over this concentration range, with a correlation coefficient (r^2) greater than 0.998. The precision of chromatographic analyses was

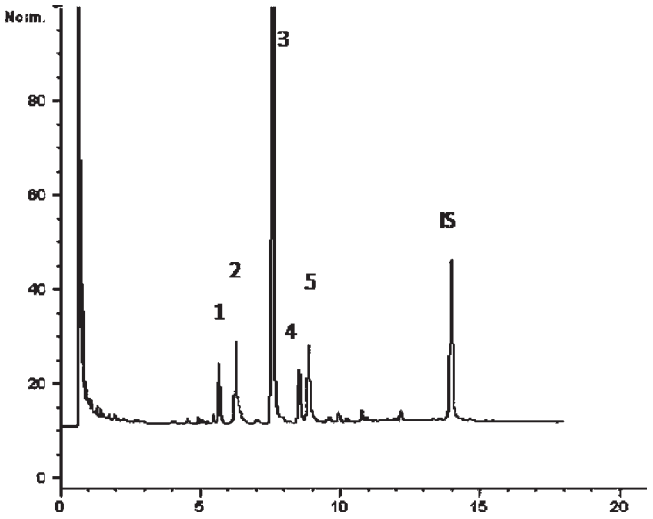

Figure 2. GC-FID chromatogram of pentacyclic triterpenes in the adult olive leaf: erythrodiol (1), uvaol (2), oleanolic acid (3), ursolic acid (4), maslinic acid (5), and 18β-glycyrrhetinic acid (IS).

evaluated by the relative standard deviation (RSD) of six replicate analyses of calibration curves in which the RSD was lower than 3%. The detection limit expressed as the mass of 18β-glycyrrhetinic acid which gave a signal 3σ above the mean blank signal was 15 μg/mL (where σ is the standard deviation of the blank signal).

The linearity in the response factor (RF) between 18β-glycyrrhetinic and oleanolic acids was studied in ethanolic solutions containing both pentacyclic triterpenoid acids in a range of concentrations (0.2−2.0 mg/mL). The ratio oleanolic/18β-glycyrrhetinic of peak areas for the same concentration of both acids (RF) was 0.715. It was not necessary to make the RF determination for the rest of analyzed terpenic compounds because we have previously found that in the same chromatographic conditions this factor was 1 between oleanolic acid and the triterpenols erythrodiol and uvaol and the ursolic and maslinic acids (*17*). The triterpenic compounds present in ethanolic extracts from olive tissues were previously identified in our laboratory by means of GC-MS using the same Rtx-65TG Crossbond capillary column and a Finnigan MAT95's (Finnigan, Bremen, Germany) high resolution mass spectrometer interfaced with a HP 5890 series II GC (*17*).

Pentacyclic Triterpenoids of the Olive Leaf. It is well established that the levels of triterpenes in the olive leaf are higher than those found in the fruit and that pentacyclic triterpenoids with amirenyl skeleton (oleanane and ursane types) are the major ones (*13*). The **Figure 2** shows an example of the chromatographic analysis of the pentacyclic triterpenoids present in the adult olive leaf. The presence of very important levels of oleanolic acid and lower amounts of the maslinic and ursolic acids and of the dialcohols erythrodiol and uvaol can be observed. **Table 1** shows that oleanolic acid represents 3.00−3.50% of the olive leaf dry weight. Maslinic acid constitutes 0.50−0.75%, and ursolic acid 0.20−0.25% approximately of the dry weight of leaf. Erythrodiol and uvaol are present in similar amounts in the range 0.05−0.15% of dry matter. On the other hand, **Table 1** also reveals that the contents of these compounds are dependent on the olive variety, confirming previous results of our laboratory (*14*, *15*) and of other authors (*19*). The "Picual" cultivar is characterized by the highest level of the oleanolic, maslinic, and ursolic acids, whereas the "Arbequina" leaf exhibits the highest quantities of triterpenic dialcohols (**Table 1**).

Table 1. Pentacyclic Triterpenes in Adult Leaves from "Picual", "Hojiblanca", and "Arbequina" Olive Cultivars[a]

	olive cultivar		
terpene [mg/g]	Picual	Hojiblanca	Arbequina
erythrodiol	0.8 ± 0.1^{a}	1.1 ± 0.1^{b}	1.5 ± 0.1^{c}
uvaol	0.7 ± 0.0^{a}	1.3 ± 0.1^{b}	1.5 ± 0.1^{c}
oleanolic acid	34.5 ± 3.1^{a}	32.6 ± 2.3^{a}	29.2 ± 1.8^{b}
ursolic acid	2.5 ± 0.2^{a}	2.2 ± 0.1^{b}	2.0 ± 0.1^{c}
maslinic acid	7.3 ± 0.5^{a}	5.3 ± 0.4^{b}	4.8 ± 0.3^{c}

[a] Results are referenced to leaf dry weight and expressed as mean ± SD of three independent samples of each variety analyzed in triplicate. (a−c) Results in a row not sharing a common letter are significantly different ($p > 0.05$).

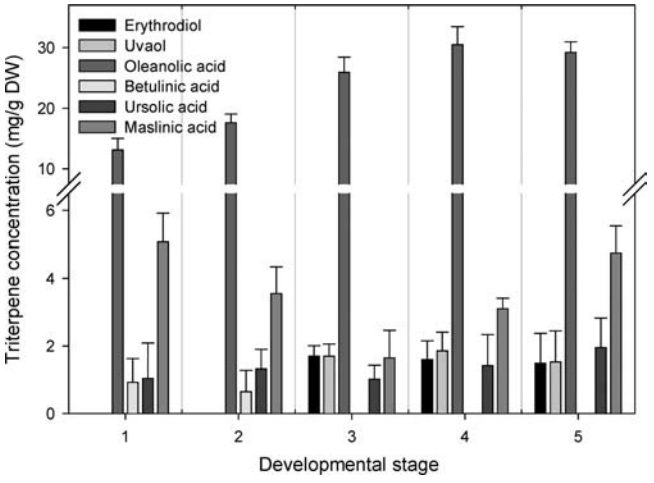

Figure 3. Evolution of pentacyclic triterpenes in the "Arbequina" olive leaf during its ontogeny. Four different developmental stages have been considered: stage 1, light-green leaves surrounding the apical bud, stage 2, leaves set in the distal area of the branch next to the apical bud with semimaximal size and bright light-green color, stage 3, leaves set in the distal third of the branch with dark-green color and semimaximal size, stage 4, leaves with definitive color and size established in the second third of the olive twigs, and stage 5, adult presenescent leaves set in the proximal extreme of the branch.

Evolution of Triterpenoids along the Leaf Ontogeny. Olive is a peculiar deciduous tree in which the change of leaves takes place progressively in a long period, which can reach and even occur over three years (*20*). For it, data reported up to the moment about triterpenoids contents in the olive leaf would represent only average values of the triterpenic composition in a determined developmental stage. It is predictable that the abundance and profile of these compounds could change during the leaf ontogeny. With this hypothesis, we analyzed the pentacyclic triterpenoids of the "Arbequina" leaf in five stages of development (**Figure 3**). First, it is observed that triterpenic fraction changes qualitatively and quantitatively along the leaf ontogeny. The triterpenic dialcohols erythrodiol and uvaol were absent in the younger phases of development (stages 1 and 2) and were clearly detected in the stages 3−5 at levels close to 1.5−2.0 mg/g DW. Oleanolic acid was the main pentacyclic triterpene in all the stages assayed, and its concentration steadily increased when the leaf ontogeny advances. The youngest organs, those around the twigs apical bud (stage 1), showed an oleanolic acid content of 13.1 ± 1.9 mg/g DW, whereas the adult leaves (stages 4 and 5) exhibited levels 2.5-fold higher. Betulinic acid only was present at low concentrations (0.5−1.0 mg/g DW) in the two earlier developmental stages assayed and followed a clear decreasing trend. Ursolic acid could be quantified in all the studied stages, and its levels did not exhibit significant modifications along the ontogeny. Finally, maslinic acid was always the second triterpenoid in abundance, and it exhibited a U-shape evolution with the highest levels in the youngest and the oldest leaves (ca. 5.0 mg/g DW).

Pentacyclic Triterpenoids of the Olive Fruit. Figure 4A shows an example of the triterpenic profile of "Arbequina" olive fruit and confirms that maslinic acid is the major pentacyclic triterpene in this drupe. In addition, a significant level of oleanolic acid was identified and, unlike the leaf, erythrodiol, uvaol, and the betulinic and ursolic acids were not detected. The **Table 2** displays the concentrations of triterpenoids found in fruits of the "Picual", "Hojiblanca", and "Arbequina" olive cultivars and corroborates that the triterpenic content in fruits is approximately 30-fold lower than that determined in olive leaves. The content of pentacyclic triterpenic compounds is dependent on the developmental stage of the fruit. The total triterpene content was higher in the unripe fruit and decreased when the ripening advanced. In the period that happens between the "green mature" stage and the "black ripe" one, triterpenoids diminished 20%, with similar decreases in the levels of the maslinic and oleanolic acids. The **Table 2** also indicates that the balance of maslinic and oleanolic acids seems to be dependent on the cultivar. Thus, in the "Picual" and "Hojiblanca" varieties, and in both ripening stages, the maslinic acid levels were ca. 2.5-fold higher than the oleanolic acid contents. In the case of "Arbequina" olive, the maslinic acid/oleanolic acid ratio was 4.0.

Distribution of Pentacyclic Triterpenoids among Olive Fruit Tissues. It has been assumed that triterpenoids are components of the olive fruit epidermis, where they represent a high percentage of the cuticle lipids (*9*, *11*). In this work, we studied the distribution of pentacyclic triterpenes among the different tissues that constitute the "Arbequina" olive fruit. The results indicate unequivocally that maslinic and oleanolic acids are exclusively located in the epidermis (**Figure 4B**). These triterpenoids were not detected in the flesh (**Figure 4C**) and seed (**Figure 4D**) of the olive drupe. Similar results have been obtained in the analysis of the woody shell of the fruit endocarp (data not shown). On the other hand, **Figure 4** also shows the presence of unidentified compounds with RTs 11−13 min. Preliminary assays realized in our lab suggest the presence of mono- and diglycerides in the ethanolic extracts. Likewise, chromatographic peaks eluting far away to 15 min would correspond to triacylglycerols. Thus, the presence of these compounds would be more prominent in ethanolic extracts of seeds that were not previously degreased with the hexane flash-treatment.

DISCUSSION

In this work, we have established a new, simple, and precise procedure for the extraction and analysis of the hydroxy pentacyclic triterpenes from olive tissues. By a two-step solid/liquid extraction with absolute ethanol, we achieved the total recovery of triterpene dialcohols and pentacyclic triterpenic acids, and by silylation of the sample and GC-FID analysis using 18β-glycyrrhetinic acid as internal standard, we carried out their identification and quantification (**Figures 2**). With these protocols, the triterpene composition of fruits and leaves from the three main Spanish olive cultivars ("Picual", "Hojiblanca", and "Arbequina") has been studied.

The results corroborate that the adult olive leaf contains very important amounts of oleanolic acid (3.0−3.5% DW), followed by significant concentrations of maslinic acid and minor levels of ursolic acid, erythrodiol, and uvaol. Data previously reported by different authors indicate that the profile and relative concentrations of pentacyclic triterpenoids in the olive leaf are dependent

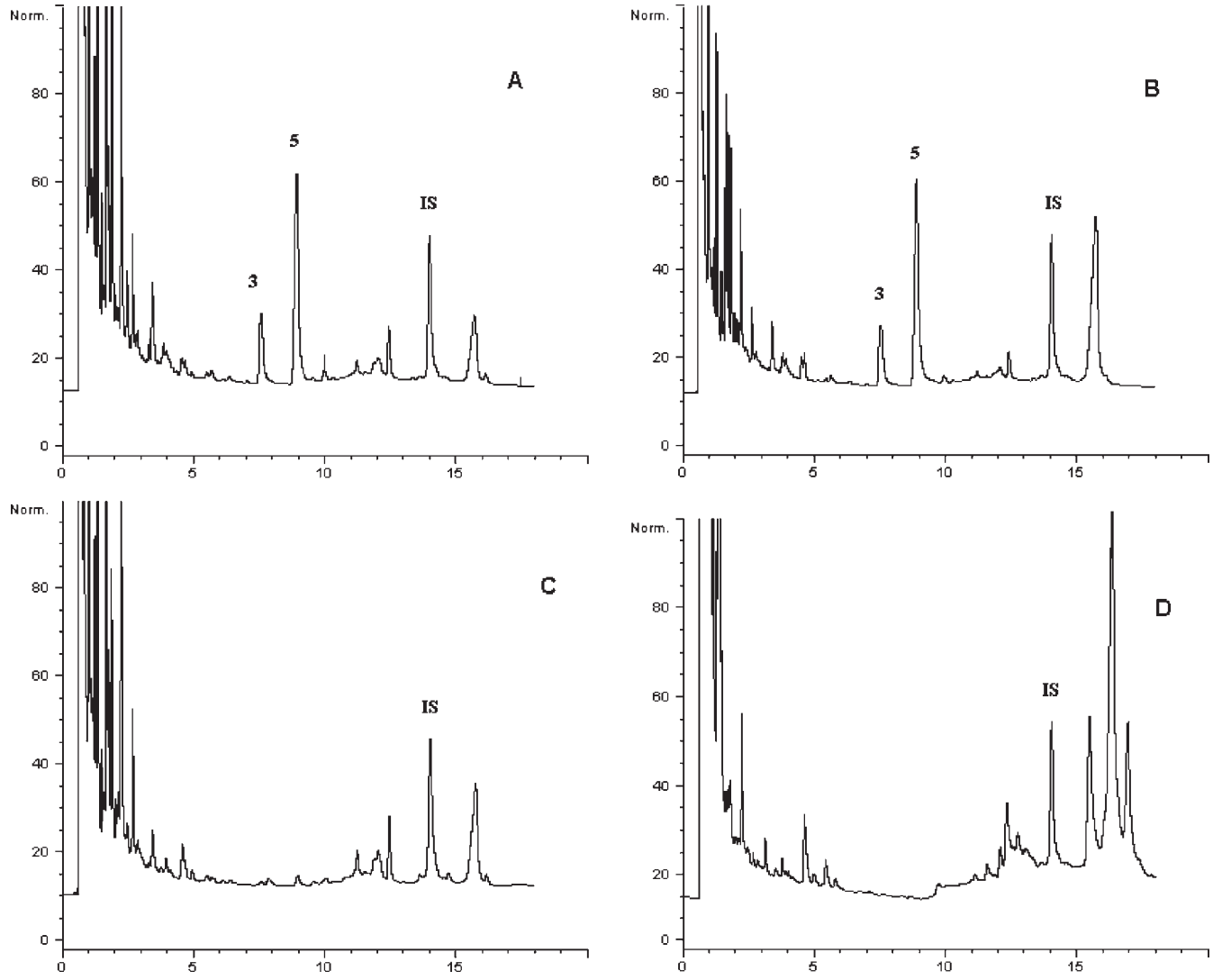

Figure 4. GC-FID chromatogram of pentacyclic triterpenes in different tissues of the "Arbequina" olive fruit: (**A**) whole fruit, (**B**) epicarp, (**C**) mesocarp, and (**D**) seed. Peaks are numbered according to **Figure 2**: oleanolic acid (3) and maslinic acid (5).

Table 2. Pentacyclic Triterpenes Detected in Fruits from the "Picual", "Hojiblanca", and "Arbequina" Olive Cultivars[a]

		CI	triterpenic acids [mg/g]	
olive cultivar	ripening stage	$(L^*(b^* - a^*))/100$	oleanolic	maslinic
Picual	green mature	33.5 ± 2.0	0.6 ± 0.0 [a]	1.5 ± 0.1 [a]
	black ripe	0.2 ± 0.5	0.5 ± 0.0 [b]	1.2 ± 0.1 [b]
Hojiblanca	green mature	34.4 ± 2.3	0.6 ± 0.0 [a]	1.6 ± 0.1 [a]
	black ripe	0.4 ± 1.8	0.5 ± 0.0 [b]	1.3 ± 0.1 [b]
Arbequina	green mature	32.8 ± 2.3	0.5 ± 0.2 [a]	1.8 ± 0.2 [a]
	black ripe	0.5 ± 1.2	0.4 ± 0.1 [a]	1.5 ± 0.1 [b]

[a] Results are referenced to fruit dry weight and expressed as mean ± SD of three independent samples of each variety analyzed in triplicate. It includes the colorimetric index (CI) used to characterize the ripening stage of fruits. (a−c) For each olive cultivar, results in a column not sharing a common letter are significantly different ($p > 0.05$).

on the tree variety. Bianchi et al. (*13*) showed that oleanolic and betulinic acids were the main pentacyclic triterpenes in the "Coratina" and "Cipressino" olive leaves, besides relevant levels of erythrodiol and uvaol. Sánchez-Ávila et al. (*19*) analyzed the leaves from the "Hojiblanca" and "Acebuche" olive cultivars and found that in both varieties the triterpene composition is marked by a high concentration of oleanolic acid (0.85−1.10% FW) and similar concentrations (0.25−0.50%) of erythrodiol, uvaol, ursolic acid, and maslinic acid. More recently, Jäger et al. (*21*) have studied the triterpenic composition of a number of plant tissues, among which they included leaves from a Greek olive collection [cultivar(s) was/were not specified]. These authors prepared extracts from the dry matter by accelerated solvent extraction with *n*-heptane and have reported values for the oleanolic acid (3.10% DW) and maslinic acid (0.18% DW) contents. In this work, we confirm that "Picual" leaf exhibited the highest levels of pentacyclic triterpenic acids, whereas "Arbequina" showed the highest relative amounts of triterpenic dialcohols (**Table 1**).

As we mentioned above, it was predictable that the abundance and profile of pentacyclic triterpenoids could change during the olive leaf ontogeny. Effectively, this study displays for the first time at our knowledge that these qualitative and quantitative changes in the triterpenic fraction occurred. We have determined that the levels of oleanolic acid steadily increased along the leaf development, while the concentration of maslinic acid exhibited a U-shape evolution (**Figure 3**). Ursolic acid content did not change significantly its moderate value throughout the leaf ontogeny, and betulinic acid only could be detected in the youngest stages (**Figure 3**). Triterpene dialcohols were produced at the most advanced developmental phases (**Figure 3**). According to these data, it could be proposed that pentacyclic triterpene biosynthesis is differentially regulated along the leaf ontogeny, favoring the

formation of the oleanyl carbocation derivatives (erythrodiol, oleanolic acid, and maslinic acid) and repressing the generation of lupenyl derivatives as betulinic acid. The ursanyl-type compounds (uvaol and ursolic acid) also experiment a moderate increase, that consisted basically in an augmentation of the uvaol contents. On the other hand, considering the evolution of the oleanane triterpenoids, it could be suggest some kind of homeostatic control of the oleanolic acid levels in the olive leaf.

This work has also exposed that in fruits of the "Picual", "Hojiblanca", and "Arbequina" cultivars the contents of pentacyclic triterpenoids represent approximately a 0.2% of the dry weight of the organs (30-fold lower than in the leaf) (**Table 2**) and that they are exclusively located in the epicarp (**Figure 4**). In these olive varieties, maslinic acid has been determined as the main triterpenoid accompanied by important quantities of oleanolic acid. Also in contrast with the leaf, other pentacyclic triterpenoids were not detected. We have observed that along with the fruit ripening the levels of maslinic and oleanolic acids decreased (**Table 2**).

Bianchi et al. (*22*) studied the composition of the epicuticular waxes of the "Coratina" olive fruit and found that the only triterpenes present were the pentacyclic triterpenoids, with oleanolic acid as the main component (70−83%). These authors also pointed that the triterpenoid composition might change during the ripening. Thus, although the hegemony of oleanolic acid was unaltered, they detected traces of erythrodiol and uvaol in the green mature fruit that were absent in more advanced stages and measured minor amounts of maslinic, ursolic, and betulinic acids in the black ripe olive. This same research group investigated the pentacyclic triterpenic acids in fruit from the "Cipressino", "Dritta", and "Leccino" olive cultivars and concluded that these fruits contained similar amounts of oleanolic and maslinic acids (*11*). In addition, these authors reported that the content of oleanolic acid in the "Cipressino" fruit diminished when the ripening advanced, whereas the level of maslinic acid increased. More recently, Stiti el al. (*12*) have studied the triterpene composition of the "Chemlali" olive fruit along its ontogeny and have reported that the complete sequence of reactions from triterpene monoalcohols to pentacyclic triterpenic acids, via triterpene diols and aldehydes, might take place in the green epicarp of the fruit. At the earlier phases of fruit growth, the authors found that oleanolic acid was the main triterpenoid followed by maslinic acid and minor amounts of the pentacyclic monoalcohols and dialcohols. Later, in more advanced steps of the development and up to the onset of fruit ripening, the contents of triterpenic intermediates progressively decreased and maslinic acid became the major triterpenoid followed by oleanolic acid. A significant decline in the content of triterpenoids was measured once the ripening began. On the other hand, Romero et al. (*23*) have just published a study on 17 cultivars intended for table olives and have corroborated that the raw fruits showed important differences in their triterpenic acids contents. The sum of maslinic and oleanolic acid ranged from 1.5 to 3.0 mg/g fruit flesh, with the concentration of maslinic acid being higher than that of oleanolic acid for all the studied varieties. In contrast, Jäger et al. (*21*) have published data about the pentacyclic triterpene presence in the olive fruit and only could detect oleanolic acid (0.21% DW).

Literature cited above displays that the available data about the profile and concentrations of the different triterpenoids in *Olea europaea* are diverse and in several cases discordant. The chemical composition of a cultivar is mainly determined by the genetic factors, but also the stage of development, geographical origin, and the environmental conditions influence that composition. In this sense, a valuable aspect of our work is that all the leaves and fruits that have been analyzed come from the same agricultural estate, where, obviously, the microclimate and the culturing conditions (soil's features, sun incidence, rainfalls, wind, altitude, pruning operations, fertilization and irrigation programs, etc.) have been identical. For this reason, the differences found among the three olive cultivars could mainly be attributable to the genetic factors.

The physiological role of pentacyclic triterpenes and triterpenoid saponins is not definitively established, although it is proposed their implication in the mechanisms of the plant defensive active responses against environmental adverse conditions and the attack of microorganisms, insects, and pests (*24*). In this sense, our laboratory has demonstrated an inverse correlation among the levels of maslinic and oleanolic acids in olive fruit and the egg-laying of *Bactrocera oleae* (*25*). On the other hand, the in situ analysis by microspectroscopy of the leaf cuticles of *Prunus laurocerasus* revealed that its cuticular waxes are arranged in distinct layers differing in triterpenoid concentrations. In fact, triterpenoids were found to accumulate in greater amounts over the guard cells relative to the pavement cells in the abaxial surface of the leaf (*26*). According to this, we suggest that oleanolic acid and the other triterpenes in the olive leaf could play a key role in the defensive strategy against pathogenic microorganisms that used the natural openings like stomata as routes of penetration.

The dramatic changes in the color, flavor, texture, and composition that a fruit experiences during its ripening are related to their physiological mission that consists in assuring the diffusion and fall to land of the seed. These sensorial and compositional changes pursue to make the fruit attractive to potential predators (birds, rodents, insects, humans). In this respect, the decline in the pentacyclic triterpenes (or triterpenoid saponins) contents during the olive fruit ripening would be consistent with this argument.

In summary, *Olea europaea*, mainly its leaf, constitutes a suitable source for the obtaining of oleanolic and maslinic acids that are increasingly demanded by the markets to explore their incorporation in functional foods, drugs, and cosmetics. Spain accumulates every year more than 1.2 million tons of this plant material which is scantly exploited and generates a serious environmental problem (*27*). The recovery of bioactive compounds of very high added value would contribute to increasing the profitability of the olive grooves.

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